EXHIBIT A

Gonadotropin-releasing hormone antagonists containing novel amino acids

G.-C. Jiang, J. Porter, C. Rivier, A. Corrigan, W. Vale and J.E. Rivier

Clayton Foundation Laboratories for Peptide Biology, The Salk Institute for Biological Studies, 10010 N. Torrey Pines Road, La Jolla, CA 92037, U.S.A.

Introduction

We recently reported that azaline B [Ac-D2Nal[1],D4Cpa[2],D3Pal[3],4Aph[5](Atz),D4Aph[6](Atz),ILys[8],DAla[10]]GnRH was among the most potent and long acting antagonists of GnRH with adequate solubility in aqueous solutions at neutral pH [1]. In order to further improve its properties, several GnRH antagonists containing novel amino acids at positions 5 (L-isomers), 6 (D-isomers) or 8 (L-isomers) have been synthesized, characterized and tested in an *in vitro* pituitary cell culture assay and in an antiovulatory assay. The synthetic amino acids (Figure 1) are D- and L-3-aminophenylalanine (3Aph), D- and L-4-thiomorpholinophenylalanine (Tmf), D and L-4-Aminomethyl- phenylalanine (4Amf), L-4-isopropyl-aminomethylphenylalanine (4IAmf), L-4-Isopropyl-amino-phenyl-alanine (4IAph) and N^{α}-methyl-4-amino-phenylalanine (NMe4Aph). Their additional distal amino groups were protected either by Fmoc or by Z except for D- and L-Tmf which have distal tertiary amino groups.

Results and Discussion

The desired phenylalanine derivatives were synthesized following five synthetic routes and their structures shown in Figure 1. D- and L-N^{α}-Boc-N^{ω}-Fmoc-3Aph [2] were prepared via condensation of 3-nitrobenzyl chloride with diethyl acetamidomalonate, followed by resolution with α-chymotrypsin, hydrogenation and Fmoc-protection of the ω-amino groups. D- and L-N^{α}-Boc-Tmf [3] were prepared via chloromethylation and bromomethylation of D- and L-N-acetyl-phenylalanine ethyl ester respectively, followed by amination (thiomorpholine), thorough hydrolysis and Boc-protection of α-amino groups. D- and L-N^{α}-Boc-N^{4}-Fmoc-4Amf were prepared with an improved method [4]. The synthesis was started from trichloro- or trifluoro-acetamidomethylation of D- and L-phenylalanine, followed by N^{α}-Boc-protection, selective deprotection of 4-amino groups by 20% sodium hydroxide in a mixture of methanol and water (v/v, 1:1) for 0.5 hr with subsequent Fmoc-protection of the exposed 4-NH_2. L-N^{α}-Boc-N^{4}-Z-4IAmf and L-N^{α}-Boc-N^{4}-Z-4IAph [5] were prepared via reductive isopropylation of L-N^{α}-Boc-4Amf and L-N^{α}-Boc-4Aph respectively and Z-protection of the resulting secondary amino groups. L-N^{α}-Boc-N^{4}-Fmoc-NMe4Aph was prepared [6] via N^{α}-methylation of N-Boc-phenylalanine, nitration, hydrogenation and Fmoc-protection of the 4-amino group.

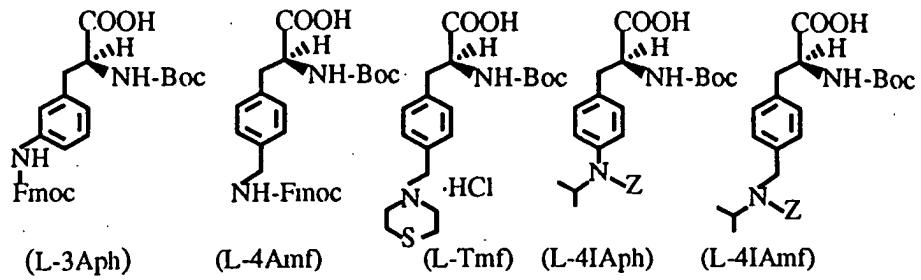

Fig. 1. Partial structure of novel phenylalanine derivatives.

Table 1 *Biological characterization of GnRH antagonists with novel amino acids at position 5, 6 and/or 8*

Ac-D2Nal[1]-D4Cpa[2]-D3Pal[3]-Ser[4]-Aaa[5]-Aaa[6]-Leu[7]-Aaa[8]-Pro[9]-DAla[10]-NH_2	*in vitro* relative potencies[a]	AOA[b]	*in vitro* histamine release[c]
1. [Lys[5](Atz),DLys[6](Atz),ILys[8]] **Azaline A**	0.23 (0.15-0.36)	2.0(1/10), 2.0(8/20)*	139 ±8.7
2. [Orn[5](Atz),DOrn[6](Atz),ILys[8]]	0.2 (0.1-0.3)	2.0(1/10)	158 ±10
3. [4Aph[5](Atz),D4Aph[6](Atz),ILys[8]] **Azaline B**	1.3 (0.8-2.1)	0.5(7/9), 1.0(0/7)	224 ±23
4. [4Amf[5](Atz),D4Amf[6](Atz),ILys[8]]		2.5(0/7)	
5. [3Aph[5](Atz),D3Aph[6](Atz),ILys[8]]	1.5 (0.9-2.8)	2.5(0/5)	17
6. [4Aph[5](Atz),D4Aph[6](Atz),4IAph[8]]		10(2/2)	
7. [4Aph[5](Atz),D4Aph[6](Atz),4IAmf[8]]	1.6 (1.0-2.6)	2.5(3/7)	
8. [NMe4Aph[5](Atz),D4Aph[6](Atz),ILys[8]] **Azaline C**	1.9 (0.85-2.2)	1.0(2/9), 2.5(0/8)	72
9. [NMe4Aph[5](Atz),D4Aph[6](Atz),4IAph[8]]		10(2/2)	
10. [NMe4Aph[5](Atz),D4Aph[6](Atz),4IAmf[8]]	1.5 (1.0-2.3)	5.0(0/6)	
11. [Tmf[5],DTmf[6],ILys[8]]		2.5(0/5)	
12. [Tmf[5],DTmf[6],Tmf[8]]		50(7/8)	
13. [Tyr[5],DPal[6],Tmf[8]]		10(0/3)	
14. [4Aph[5](Ser),D4Aph[6](Ser),ILys[8]]		1.0(4/5)	
15. [4Aph[5](DSer),D4Aph[6](DSer),ILys[8]]		2.5(2/5)	
16. [4Aph[5](Ac-Ser),D4Aph[6](Ac-Ser),ILys[8]]		1.0(4/7)	
17. [4Aph[5](Ac-DSer),D4Aph[6](Ac-DSer),ILys[8]]	0.5 (0.4-0.7)	1.0(2/7)	

[a]Relative to [Ac-Δ^3Pro[1],DFpa[2],DTrp[3,6]]GnRH = 1.0. [b]AOA = antiovulatory assay: dosage in micrograms/rats (rat ovulating/total), peptides were dissolved in ca.1% DMSO/saline or (*) in corn oil. [c]ED_{50} ± SEM, µg/mL. ED_{50} for [Ac-DNal[1],DFpa[2],DTrp[3],DArg[6]]GnRH (internal standard) was 0.17 ± 0.01 µg/mL. Peptides fully active at 10 µg, 2.5 µg and 1.0 µg were only partially active at 5.0 µg, 1.0 µg or 0.5 µg, respectively.

The difference between peptide **1** and **2** is the shortening of the side chains at

positions 5 and 6 with no significant effect on biological potencies. Peptide **3** on the other hand is significantly more potent in the AOA and in the castrated male rat assay where it was found to be considerably longer acting than peptide **1** [1]. We suggested that this difference resulted from the presence of the aromatic ring which shielded access to the backbone against enzymatic hydrolysis. Introduction of a methylene group on the para position of the phenyl ring (peptide **4**) and introduction of the triazolyl function on meta-aminophenylalanines (peptide **5**) at positions 5 and 6 resulted in a significant lowering of potency in the AOA. Because ILys at position 8 was recognized to maintain AOA potency while contributing to a major reduction of the histamine releasing activity, we investigated the possibility to substitute it by an aromatic containing amino acid such as 4IAph or 4IAmf (peptides **6** and **7**). In both cases, considerable loss of potency resulted from these substitutions. We also synthesized three analogs (**8-10**) containing NMe4Aph in position 5 based on the observation of Haviv et al. [7] that N-methylation at position 5 conferred increased solubility. Analog (**8**) was more soluble, however, significantly less potent in the AOA and furthermore released histamine at a significantly lower concentration than (**3**). Introduction of IAph and IAmf at position 8 of the NMe substituted Azaline B (**9, 10**) also resulted in further loss of potency. Another approach to increasing water solubility was to increase basicity at positions 5, 6 and 8 (peptides **11-13**) by the introduction of one or several Tmf residue(s) which encompass a thiomorpholino moiety. Analogs **12** and **13** were significantly less potent while **11** was fully potent at 2.5 µg in the AOA. Finally, we investigated the possibility of increasing solubility by the introduction of a D- or L-serine (or an Acetyl-D- or L-serine) on the 4Aph side chain at positions 5 and 6 of Azaline B (peptides **14-17**). These peptides were also less potent than Azaline B.

Acknowledgements

This work was supported in part by NIH under Contract N01-HD-9-2903, the Hearst Foundation and the World Health Organization (WHO) Research Training Grant funded by the United Nations Fund for Population Activities (UNFPA) under Project CPR/90/P.25. We acknowledge the outstanding technical contributions of Ron Kaiser, Laura Cervini, Charleen Miller and Duane Pantoja. We thank Dr. A.G. Craig for the mass spectra.

References

1. Rivier, J.E., Porter, J., Hoeger, C., Theobald, P., Craig, A.G., Dykert, J., Corrigan, A., Perrin, M., Hook, W.A., Siraganian, R.P., Vale, W. and Rivier, C., J. Med. Chem., 35 (1992) 4270.
2. Porter, J., Dykert, J. and Rivier, J., Int. J. Peptide Protein Res., 30 (1987) 13.
3. He, B., Liu, K.-L. and Shaobo, X., Chinese Sci. Bull., 22 (1988) 1712.
4. Stokker, G.E., Hoffman, W.F. and Homnick, C.F., J. Org. Chem., 58 (1993) 5015.
5. Augustine, R.B., Catalytic Hydrog., M. Dekker, Inc., New York, NY, U.S.A., 1965, p.102.
6. Cheung, S.T. and Benoiton, N.L., Can. J. Chem., 55 (1977) 906.
7. Haviv, F., Fitzpatrick, T.D., Nichols, C.J., Swenson, R.E., Mort, N.A., Bush, E.N., Diaz, G., Nguyen, A.T., Holst, M.R., Cybulski, V.A., Leal, J.A., Bammert, G., Rhutasel, N.S., Dodge, P.W., Johnson, E.S., Cannon, J.B., Knittle, J. and Greer, J., J. Med. Chem., 36 (1993) 928.